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Dual Enzymatic Routes to L-Tyrosine and L-Phenylalanine via Pretyrosine in *Pseudomonas aeruginosa**

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Pretyrosine, an intermediate of L-tyrosine biosynthesis in blue-green algae, was found to be enzymatically formed and utilized in *Pseudomonas aeruginosa*. The enzymology and regulation of aromatic biosynthesis were re-evaluated in the context of these new findings. Four species of aromatic aminotransferase were separated and partially purified. Each was reactive with prephenate, phenylpyruvate, and 4-hydroxyphenylpyruvate. Molecular weights of aminotransferases DE I and HA I were 70,000, whereas aminotransferases HA II and HA III had molecular weights of 200,000. L-Glutamate was the best amino donor reactant with aminotransferase DE I, whereas L-leucine was best with the remaining three aminotransferases.

Gel filtration, DEAE-cellulose chromatography, and hydroxylapatite chromatography did not separate prephenate dehydrogenase activity from pretyrosine dehydrogenase activity (M_r = 140,000). Pretyrosine dehydratase, a previously undescribed enzyme activity, converts pretyrosine to L-phenylalanine in a reaction that is analogous to the conversion of prephenate to phenylpyruvate (prephenate dehydratase). Pretyrosine dehydratase is resolved by hydroxylapatite chromatography into three subforms with similar properties and identical molecular weights (M_r = 80,000). Pretyrosine dehydratase is also reactive with prephenate, functioning *in vitro* as prephenate dehydratase.

Chorismate mutase II (M_r = 27,000) and pretyrosine dehydratase are unregulated. The bifunctional protein, chorismate mutase/prephenate dehydratase (M_r = 134,000), does not appear to be an aggregate of chorismate mutase II and pretyrosine dehydratase. Both activities of the bifunctional protein are not only inhibited by L-phenylalanine, but prephenate dehydratase is activated by L-tyrosine (alteration of the apparent K_m). Prephenate/pretyrosine dehydrogenase is feedback inhibited by L-tyrosine. Activity of 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthetase is not influenced by pretyrosine. The dual enzymatic pathways to either L-phenylalanine or L-tyrosine provide an enzymological basis for explanation of the failure of routine mutagenesis procedures to produce auxotrophs with absolute requirements for either amino acid.

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In our initial enzymological studies of aromatic biosynthesis in *Pseudomonas aeruginosa* (1), we assumed that the aromatic pathway was identical with that previously established in such organisms as *Escherichia coli* and *Bacillus subtilis* (2) since all enzyme activities sought were readily identified in crude extracts of *P. aeruginosa*. The presence of an alternative biosynthetic pathway to L-tyrosine, the pretyrosine pathway, was recently described in species of blue-green algae (3). The pretyrosine pathway enzymes for tyrosine biosynthesis were later found in *P. aeruginosa*, even though the reactions comprising the 4-hydroxyphenylpyruvate pathway were also present (4). This paper shows that an enzymatic sequence to L-phenylalanine via pretyrosine also exists, in addition to the presence of the phenylpyruvate pathway to L-phenylalanine. The newly identified pretyrosine sequences to L-phenylalanine and L-tyrosine are shown below.

Regardless of the evolutionary and physiological significance of such apparent metabolic extravagance (a topic which has been considered (5, 6) elsewhere), the newly recognized enzymes permit an appreciation of the basis for a number of unorthodox observations in the *P. aeruginosa* system. These include: (*a*) inability to isolate tightly blocked auxotrophs for phenylalanine or tyrosine; (*b*) the general resistance of *P. aeruginosa* to growth inhibitory effects of aromatic analogues that are potent antimetabolites for other microorganisms (7); and (*c*) a variety of anomalous enzymological results in crude extracts. All of the latter phenomena can now be accounted for by the presence in *P. aeruginosa* of enzymological sequences to L-tyrosine and L-phenylalanine via pretyrosine in addition to the better known phenylpyruvate and 4-hydroxyphenylpyruvate sequences.

MATERIALS AND METHODS

Microbiological Aspects

Strain 1 of *Pseudomonas aeruginosa* was obtained from B. W. Holloway (8). Cultures were grown at 37° in a minimal salts-glucose medium (7). The cells were harvested by centrifugation during the late exponential phase of growth.

Preparation of Crude Extracts

Cell-free extracts were prepared in 50 mM Tris·HCl, pH 7.9, as previously described (9).

Aminotransferase Assays

When aminotransferase activities were assayed with 2-ketoglutarate:phenylalanine or 2-ketoglutarate:tyrosine substrate combina-

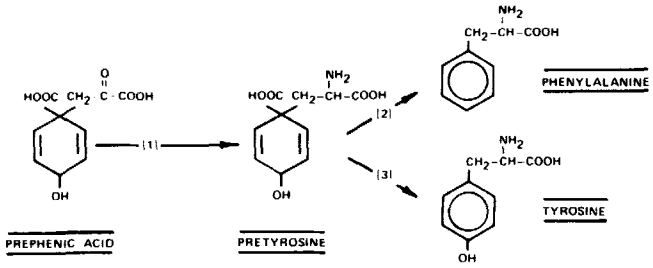


FIG. 1. Enzymes labeled [1], [2], and [3] denote prephenate transaminase, pretyrosine dehydratase, and pretyrosine dehydrogenase, respectively.

tions, the following spectrophotometric assay was used. The reaction mixture (200 μl) containing 50 mM Tris·HCl buffer (pH 7.9), 0.025 mM pyridoxal-5'-phosphate, 2.5 mM 2-ketoglutarate, and 10 mM phenylalanine or tyrosine was incubated at 37° for 20 min. A 0.8-ml amount of 2.5 N NaOH was then added, and the absorbance of phenylpyruvate at 320 nm or of 4-hydroxyphenylpyruvate at 331 nm was measured as appropriate.

Alternatively, in some experiments a more rigorous procedure employing radiolabeled amino acids was used. A method previously described for the assay of 2-ketoglutarate/tyrosine transaminase (10) was used with some modifications. This method allowed a broader appraisal of aminotransferase reactivity with various combinations of substrate; it also was used for the independent confirmation of assay results obtained with the spectrophotometric procedure where interference owing to the presence of other enzyme activities was possible. Reaction mixtures (100 μl) contained 50 mM Tris·HCl (pH 7.9), 25 μg of bovine serum albumin, 0.025 mM pyridoxal-5'-P, 0.25 mM L-[^{14}C]glutamate (0.125 μCi) or 0.25 mM L-[^{14}C]leucine (0.125 μCi), 5 mM keto acid, and enzyme. The keto acids tested included phenylpyruvate, 4-hydroxyphenylpyruvate, prephenate, oxalacetate, 2-ketoisocaproate, and 2-ketoglutarate. After incubation at 37° for 20 min the reaction mixture was acidified by addition of 0.3 ml of 1 N HCl. The radioactive keto acid product was then extracted in 1 ml of ethyl acetate:toluene (4:1). A 200-μl sample of the organic phase was then transferred to 10 ml of Aquasol in a vial for measurement of radioactivity in a scintillation counter.

Other Analytical Procedures

Chorismate Mutase—A reaction mixture (200 μl) containing 50 mM Tris·HCl (pH 7.9), 1 mM chorismate, and enzyme was incubated at 37° for 20 min. Any prephenate formed was then converted to phenylpyruvate by incubating the reaction mixture at 37° for 15 min after addition of 0.1 ml of 1 N HCl. The absorbance of phenylpyruvate at 320 nm was measured after addition of 0.7 ml of 2.5 N NaOH, and extinction coefficient of 17,500 was used for calculations (11).

Prephenate Dehydrogenase and Pretyrosine Dehydrogenase—Reaction mixtures (200 μl) contained 50 mM Tris·HCl (pH 7.9), 0.5 mM NAD, enzyme, and 0.5 mM prephenate or pretyrosine. The formation of NADH was continuously followed (12) by use of an Aminco-Bowman spectrophotofluorometer (excitation at 340 nm, emission at 460 nm).

Prephenate Dehydratase—Reaction mixtures (200 μl) contained 1 mM prephenate, 50 mM Tris·HCl (pH 7.9), and enzyme. After incubation at 37° for 20 min, 0.8 ml of 2.5 N NaOH was added, and phenylpyruvate was measured spectrophotometrically at 320 nm.

Pretyrosine Dehydratase—The reaction mixture (100 μl) contained 2.0 mM pretyrosine, enzyme, and 50 mM Tris·HCl (pH 7.9). After incubation at 37° for 60 min the reaction was stopped by placing the tubes in an ice bath. Assay results were confirmed through the use of two methods. (*a*) The mixture was spotted on thin layer plates (Eastman Chromogram cellulose, without fluorescent indicator). The plates were developed with a 1-propanol:water (70:30) solvent (adjusted to pH 9.0 with pyridine) for 3 to 4 h to separate the phenylalanine product from the pretyrosine substrate. One-half of the plate containing pretyrosine and phenylalanine standards was sprayed with ninhydrin for visualization. The position of phenylalanine on the other half of the plate was then determined by comparison with the ninhydrin-treated half. Phenylalanine was eluted from the plate in 1 ml of deionized water. The concentration of phenylalanine was then determined by fluorometric assay (Sigma Technical Bulletin No. 60F). (*b*) In the second procedure for assay of pretyrosine dehydratase, a 100-μl reaction mixture containing 0.2 M potassium phosphate buffer (pH 7.5), 2 mM pretyrosine, and enzyme was incubated at 37° for 60 min. The reaction was stopped by moving the reaction vessels to an ice bath. A 20-μl sample was withdrawn for the assay of phenylalanine concentration. The fluorometric assay (Sigma Technical Bulletin No. 60F) was modified by replacing the succinate buffer (pH 5.0) with a 0.2 M potassium phosphate buffer (pH 7.5). At neutral pH, nonenzymatic conversion of pretyrosine to phenylalanine (2) does not occur in the time frame involved. Hence, the method allows the measurement of phenylalanine without previous separation (*e.g.* thin layer separation) from the pretyrosine substrate. Control experiments established that pretyrosine produced only about 7% of the fluorescence as phenylalanine on a molar basis under these conditions. This assay is suitable with partially purified enzyme preparations and pretyrosine preparations that are free of extraneous aromatic compounds.

3-Deoxy-D-arabino-heptulosonate-7-P Synthetase—The assay procedures of Calhoun *et al.* (1) were used.

Determination of Protein Concentrations—The relative concentration of protein eluted in fraction bands taken from chromatography columns was monitored by absorbance at 280 nm. Protein concentrations of enzyme preparations were determined by the method of Lowry *et al.* (13).

Enzyme Fractionation Procedures

DEAE-Cellulose Chromatography—A crude extract containing 100 mg of protein was applied at 4° to a DE52 (Whatman) column (1.5 × 20 cm) equilibrated with 50 mM Tris·HCl, pH 7.9, and then washed with 100 ml of the buffer. The wash effluent containing unbound protein was collected in 2.2-ml fractions. The remaining protein was eluted with a linear gradient of NaCl, one reservoir containing 150 ml of Tris·HCl buffer and the other 150 ml of Tris·HCl buffer plus 0.5 M NaCl. Fractions of 2.2-ml volume were collected.

Hydroxylapatite Chromatography—Appropriate fractions eluted from DEAE-cellulose were pooled and concentrated to 4 ml by means of an Amicon Diaflo cell (PM 10 membrane). Pyridoxal-5'-P was added to a final concentration of 0.1 mM. The concentrate was applied to a Bio-Gel HTP (Bio-Rad) column (1.5 × 6 cm) equilibrated with 0.01 M potassium phosphate buffer, pH 7.0, and the effluent was collected in 2.2-ml fractions. Column-bound protein was then eluted with a 300-ml linear gradient of 0.01 M to 0.2 M potassium phosphate buffer, pH 7.0. Fractions of 2.2-ml volume were collected at 4°.

Molecular Weight Estimation by Gel Filtration

Molecular weights were estimated by the method of Andrews (14) using a Sephadex G-200 column (1.5 × 60 cm) equilibrated with 50 mM Tris·HCl buffer, pH 7.9. The column was calibrated with the following molecular weight standards: aldolase (158,000), ovalbumin (45,000), chymotrypsinogen A (25,000), and ribonuclease A (13,700). The void volume was determined with blue dextran. Preparations of enzyme (1 ml each), partially purified as indicated in the text, were passed through the column individually in separate runs.

Biochemicals

Protein standards for molecular weight determination were obtained as a calibration kit (NO DN 01) from Pharmacia Fine Chemicals. Amino acids, keto acids, pyridoxal-5'-P, NAD, NADP, erythrose-4-P, phosphoenolpyruvate, 2-amino-2-hydroxymethyl-1,3-propanediol (Trizma) buffer, and Sephadex G-200 were obtained from Sigma Chemical Co. DE52 cellulose and hydroxylapatite (Bio-Gel HTP) were obtained from Whatman and Bio-Rad, respectively.

L-[^{14}C]glutamate (270 mCi/mmol) and L-[^{14}C]leucine (324 mCi/mmol) purchased from Amersham/Searle were purified by an organic solvent extraction method (10) before use. Aquasol was purchased from New England Nuclear. Other chemicals of commercial origin were of the highest grade available.

Potassium pretyrosine and the barium salt of prephenate were isolated from an aromatic mutant of *Neurospora crassa* which accumulates these compounds in the culture medium (15). Barium prephenate was converted to the potassium salt with K_2SO_4 prior to use. The pretyrosine preparation thus obtained was free of chorismate, prephenate, phenylpyruvate, 4-hydroxyphenylpyruvate, and amino acids. The concentration of pretyrosine was determined indirectly by conversion to phenylalanine at acid pH followed by fluorometric assay of the phenylalanine formed (Sigma Technical Bulletin No. 60F). Chorismate was isolated from the accumulation medium of *Enterobacter aerogenes* 62-1 and purified as the free acid (16).

RESULTS

Multiplicity of Aromatic Aminotransferases – Aminotransferase activities for aromatic substrates were initially separated into two fractions through the use of DEAE-cellulose chromatography (Fig. 2, *left*). Each aminotransferase was reactive (using glutamate as an amino group donor) with prephenate, 4-hydroxyphenylpyruvate, or phenylpyruvate, as well as with phenylalanine or tyrosine (using 2-ketoglutarate as an amino group acceptor). Although the early and late eluting peaks of activity (denoted DE I and DE II, respectively) exhibited broadly overlapping specificities, substantial differences in relative preference for amino-keto acid pairs are apparent from inspection of Fig. 2. The possibility was considered that the reactivity of aminotransferase DE I with prephenate might only be apparent, owing to the possible enzymatic formation during assay of another keto acceptor (phenylpyruvate) by pretyrosine dehydratase, an enzyme that is reactive with prephenate and which contaminates DE I fractions. Therefore, the pooled freactions corresponding to aminotransferase DE I were applied to a hydroxylapatite column (Fig. 2, *right*). All pretyrosine dehydratase was retained by the resin, while aminotransferase DE I eluted freely in the wash fractions. This further purified preparation of aminotransferase, now free of pretyrosine dehydratase, maintained function as prephenate aminotransferase. The ability of aminotransferase DE I to transaminate prephenate was readily confirmed by thin layer identification of the pretyrosine reaction product (see "Materials and Methods").

Unlike aminotransferase DE I, aminotransferase DE II eluted from DEAE-cellulose with an asymmetrical profile, suggestive of a mixture of several enzymes. When the pooled fractions corresponding to DE II aminotransferase activity in Fig. 2 were applied to a hydroxylapatite column (as illustrated in Fig. 3), three aminotransferase activities of overlapping substrate specificity were resolved. Aminotransferase HA I passed freely through the column and eluted in the wash. The remaining aminotransferase activity eluted in a gradient of phsophate buffer with an activity profile revealing two additional aminotransferases, designated HA II and HA III. Aminotransferases DE I, HA I, HA II, and HA III do not appear to be interconvertible species of enzyme in equilibrium mixture, since each of the four enzymes faithfully mimicked its original elution position and integrity as a single species following rechromatography on DEAE-cellulose or hydroxylapatite. Additionally, when each aminotransferase was individually passed through a Sephadex G-200 column, it eluted with a sharp symmetrical profile. The molecular weights of the four aminotransferases were estimated as indicated in Fig. 4.

Specific activities of the four aminotransferases with var-

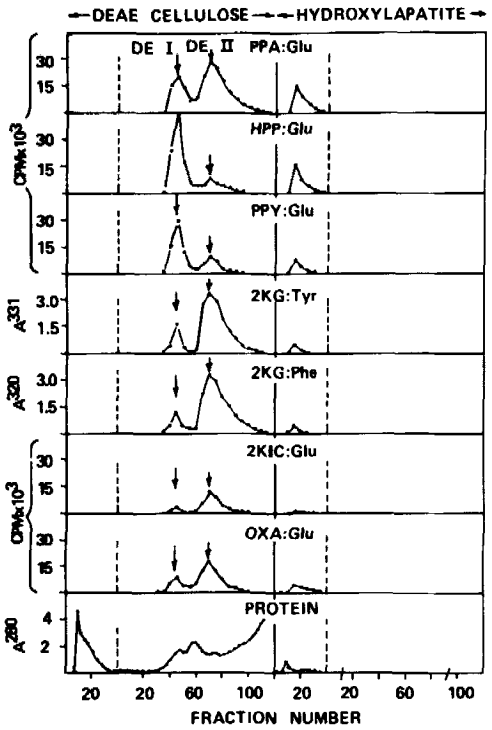


FIG. 2. Resolution of enzyme DE I from other aminotransferases and pretyrosine dehydratase. *Left*, a crude extract was chromatographed on DEAE-cellulose as described under "Materials and Methods," and eluates were assayed for aminotransferase activity using the substrate combinations indicated. Fractions to the left of the *vertical dotted line* contain proteins that elute in the wash volume. Spectrophotometric assays for phenylpyruvate or 4-hydroxyphenylpyruvate were used with 2-ketoglutarate, while [^{14}C]glutamate was used in the remaining assays (see "Materials and Methods"). The total activities per fraction per min are plotted against fraction number. Absorbance values of 1.0 correspond to 50 nmol of 4-hydroxyphenylpyruvate at 331 nm and 57 nmol of phenylpyruvate at 320 nm. An ordinate value of 1000 cpm represents 3.14 nmol of [^{14}C]2-ketoglutarate formed. *Right*, Fractions 33 to 55 (aminotransferase DE I) eluted from DEAE-cellulose were pooled, concentrated to 4 ml, and chromatographed on hydroxylapatite as described under "Materials and Methods." Eluate fractions (including 40 fractions of the wash) were assayed as before with DEAE-cellulose eluate fractions. *PPA*, prephenate; *HPP*, 4-hydroxyphenylpyruvate; *PPY*, phenylpyruvate; *2KG*, 2-ketoglutarate; *2KIC*, 2-ketoisocaproate; *OXA*, oxaloacetate; *Glu*, glutamate; Tyr, tyrosine; *Phe*, phenylalanine.

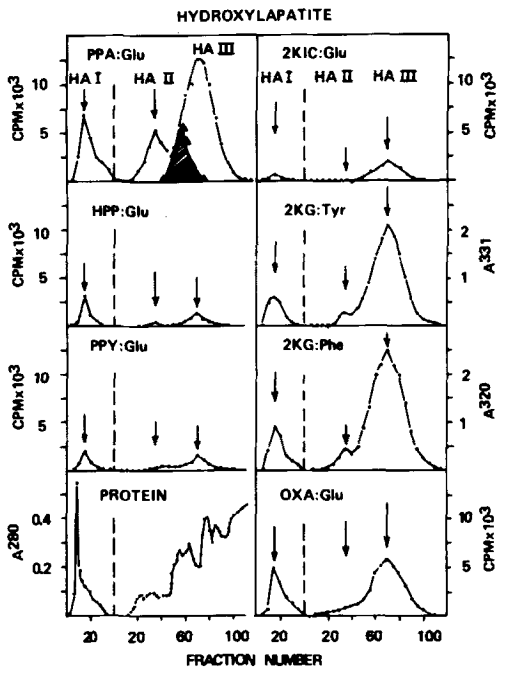


FIG. 3. Resolution of aminotransferases HA I, HA II, and HA III from DE II proteins via hydroxylapatite chromatography. Fractions 60 to 100 (DE II) from the experiment shown in Fig. 2 (*left*) were pooled, concentrated to 4.0 ml, and applied to hydroxylapatite as described in under "Materials and Methods." Abbreviations and ordinate designations are given under Fig. 2. The *shaded area* depicted in the upper left sector indicates the position of the bifunctional protein, chorismate mutase/prephenate dehydratase. The chorismate mutase and prephenate dehydratase activities in the peak fraction of the bifunctional protein were 0.35 and 0.61 absorbance at 320 nm per min per fraction, respectively.

ious substrate combinations are compared in Table I. Each aminotransferase reacts with prephenate, 4-hydroxyphenylpyruvate, or phenylpyruvate, each utilizing glutamate or leucine as an amino group donor. Aminotransferase DE I exhibits a distinctive preference for glutamate, unlike the remaining three enzymes which are more reactive with leucine; the DE I and HA III aminotransferases provide an especially striking contrast in this respect. The reactivities of all four aminotransferases with prephenate compare favorably with those of 4-hydroxyphenylpyruvate or phenylpyruvate. Aminotransferase DE I is readily inactivated at 60° while the HA II and HA III enzymes are stable for at least an hour at 60° (Fig. 5). The reason for the apparent initial activation of aminotransferase HA II at 60° is unknown. Aminotransferase HA I displays an intermediate inactivation curve, exhibiting a half-life of 70 min (compared to a half-life of 13 min at 60° for aminotransferase DE I).

Prephenate/Pretyrosine Dehydrogenase—Prephenate and pretyrosine dehydrogenase activities eluted with coincident profiles from DEAE-cellulose (Fig. 6, *left*). Fractions 65 to 85

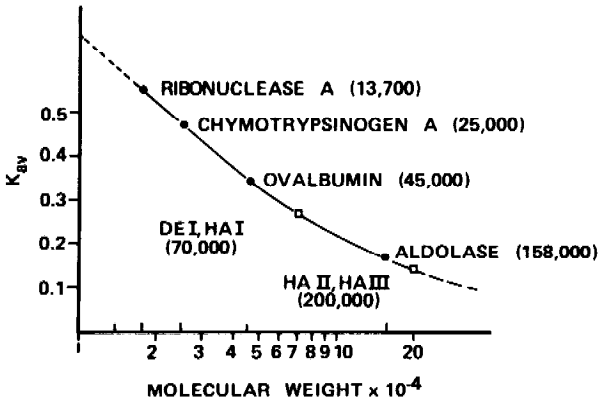


FIG. 4. Estimation of molecular weights for aromatic aminotransferases by gel filtration. A column of Sephadex G-200 was prepared and calibrated as described under "Materials and Methods." Aminotransferase DE I was obtained from the experiment shown in Fig. 2 (*right*). Aminotransferases HA I, HA II, and HA III were obtained from the experiment described under Fig. 3. In each column run, 1.0 ml of enzyme preparation was loaded on the column. Activities were located by the spectrophotometric assay described under "Materials and Methods," using 2-ketoglutarate and either phenylalanine or tyrosine as substrates. The ordinate values are expressed as $K_{av} = (v_e - v_0)/(v_t - v_0)$ where v_e = elution volume of the protein, v_0 = void volume, and v_t = total bed volume.

TABLE I

Substrate specificities of aromatic aminotransferases

The radioisotopic assay described under "Materials and Methods" was used.

Keto acceptor substrate (5 mM)	Amino donor substrate (0.25 mM)	Aminotransferase species[a] (specific activity[b])			
		DE I	HA I	HA II	HA III
		nmol/min/mg			
Phenylpyruvate	Glutamate	1.73	0.24	0.05	0.12
	Leucine	0.03	1.75	0.94	15.3
4-Hydroxyphenyl-pyruvate	Glutamate	2.24	0.30	0.09	0.31
	Leucine	0.03	0.13	0.90	10.2
Prephenate	Glutamate	0.77	0.41	0.27	0.73
	Leucine	0.04	1.28	1.85	11.8
2-Ketoglutarate	Glutamate	2.32	0.55	0.41	3.26
	Leucine	0.11	3.44	0.34	15.2
2-Ketoisocaproate	Glutamate	0.17	0.06	0.02	0.28
	Leucine	0.03	1.19	0.93	12.5

[a] Aminotransferase DE I was partially purified from DEAE-cellulose and hydroxylapatite as described in Fig. 2. Aminotransferases HA I, HA II, and HA III were separated via hydroxylapatite chromatography as detailed under Fig. 3.

[b] All four aminotransferase preparations were stable, and appropriate calculations were made to convert activities measured in partially purified preparations back to rough estimates of specific activities in crude extracts originally prepared from whole cells.

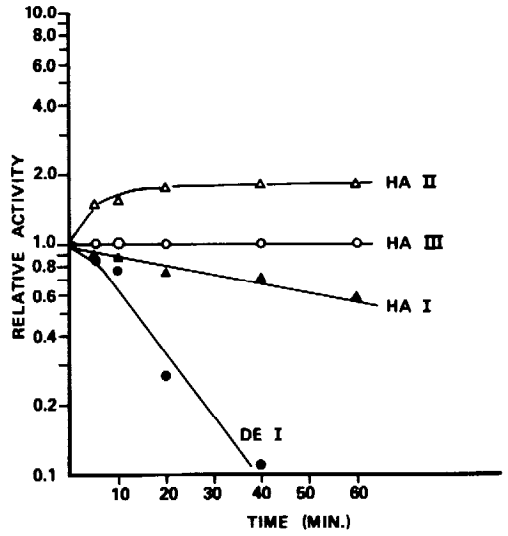


FIG. 5. Differential heat inactivation of aromatic aminotransferases. Reaction mixtures containing partially purified enzyme, 0.45 mg/ml of bovine serum albumin, 0.1 mM of pyridoxal-5'-P, and 0.05 mM Tris·HCl buffer, pH 7.9, were incubated at 60°. A 50-μl sample was withdrawn at each indicated time and assayed for phenylpyruvate aminotransferase activity. The radioisotopic method with [^{14}C]glutamate and phenylpyruvate (see "Materials and Methods") was used. Aminotransferase DE I was recovered from the hydroxylapatite step described under Fig. 2. The remaining three aminotransferases were obtained following hydroxylapatite chromatography, as described under Fig. 3. Similar experimental results were obtained when either prephenate or 4-hydroxyphenylpyruvate was substituted for phenylpyruvate as the keto acid substrate.

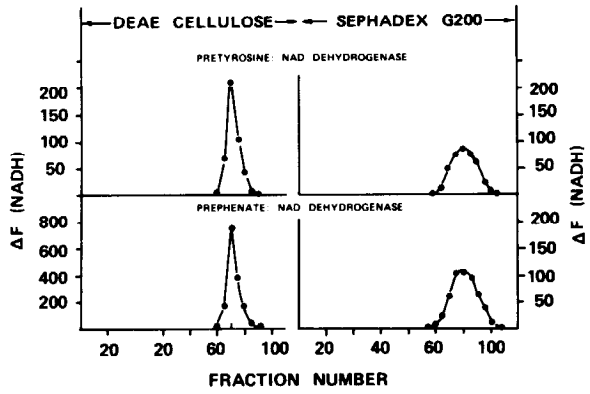


FIG. 6. Co-purification of pretyrosine dehydrogenase and prephenate dehydrogenase activities. *Left*, a crude extract containing 100 mg of protein was applied to a column of DEAE-cellulose as described under "Materials and Methods." Eluate fractions were assayed for NAD-dependent dehydrogenase activity with either prephenate or pretyrosine. Activities are expressed on the ordinate scale as fluorescence units per fraction (2.2 ml) per min. One nanomole of NADH produced corresponds to 20 fluorescence units. *Right*, Fractions 65 to 85 eluted from DEAE-cellulose (as shown on left) were pooled, concentrated to 2.0 ml (Amicon Diaflo cell, PM 10 membrane), and applied to a Sephadex G-200 column (2 × 60 cm). The column was equilibrated and eluted with 50 mM Tris·HCl buffer, pH 7.9. Eluate fractions of 1.0 ml were collected at 4°.

TABLE II

Molecular weight estimates for key enzymes of aromatic biosynthesis

Enzyme	Molecular weight	Reference citation
3-Deoxy-D-*arabino*-heptulosonate 7-phosphate synthetase	52,000	(1)
Chorismate mutase II	27,000	This paper
Chorismate mutase/prephenate dehydratase	134,000	(1)
Anthranilate synthetase		
Aminase subunit	64,000	(17)
Glutamine-binding subunit	18,000	(17)
Pretyrosine dehydratase[a]		
Species A	80,000	This paper
Species B	80,000	This paper
Species C	80,000	This paper
Prephenate/pretyrosine dehydrogenase[b]	140,000	This paper
Aminotransferase DE I	70,000	This paper
Aminotransferase HA I	70,000	This paper
Aminotransferase HA II	200,000	This paper
Aminotransferase HA III	200,000	This paper

[a] Synonymous with prephenate dehydratase II, molecular weight 76,000 (1).

[b] Refs. 1 and 9 provide molecular weight estimates of 120,000 and 150,000, respectively.

TABLE III

Effect of p-chloromercuribenzoate on prephenate and pretyrosine dehydrogenase activities

pCMB[a]	Relative activity[b]	
	Prephenate dehydrogenase[c]	Pretyrosine dehydrogenase[c]
	mM	
0	100	100
0.05	100	22
0.10	100	20
0.20	91	18
0.30	91	6
0.50	79	
0.75	91	
1.00	91	

[a] pCMB was added to a sample of enzyme to give the concentration indicated and incubated 5 min at 37° prior to initiation of the reaction with NAD and substrate.

[b] Activities measured in the absence of pCMB were assigned a relative value of 100. Relative values of 100 for prephenate and pretyrosine dehydrogenase activities are 0.17 and 0.23 nmol/min/25 μl of partially purified enzyme.

[c] The partially purified preparation of enzyme was eluted from Sephadex G-200 as described under Fig. 4.

were combined, concentrated to 4 ml by means of an Amicon Diaflo cell (PM 10 membrane), and passed through Sephadex G-200 (see "Materials and Methods"). Again, both activities co-purified (Fig. 6, *right*), eluting at a molecular weight position of 140,000 (Table II). The enzyme(s) utilized NAD, but not NADP, as an electron acceptor, whether pretyrosine or prephenate was used as the substrate.

The sulfhydryl reagent, pCMB,[1] exerted dramatic selectivity in its effect upon the two dehydrogenase activities. pCMB inhibited the activity of pretyrosine dehydrogenase almost entirely at concentrations that did not affect the activity of prephenate dehydrogenase (Table III). pCMB inhibition was reversible by 2-mercaptoethanol (data not shown).

[1] The abbreviation used is: pCMB, p-chloromercuribenzoate.

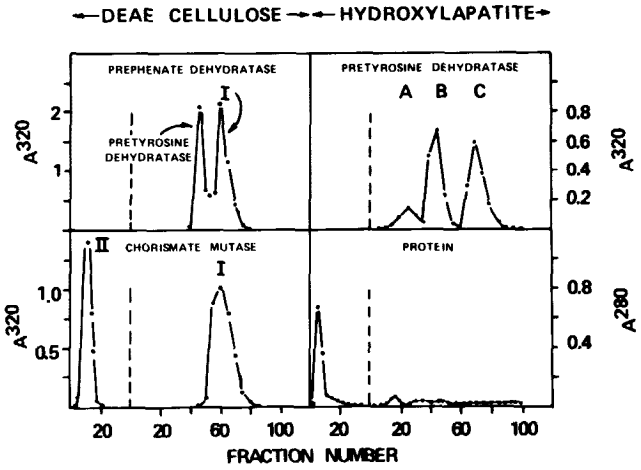


FIG. 7. Resolution of two species of chorismate mutase and three subforms of pretyrosine dehydratase. Crude extract was fractionated on DEAE-cellulose as described under "Materials and Methods." As previously shown (1) chorismate mutase I co-exists as a bifunctional protein with prephenate dehydratase, and these activities co-elute at Fraction 60 (*left*). Another protein, chorismate mutase II, passes through DEAE-cellulose without retardation. Pretyrosine dehydratase, eluting in Fraction 47 (*left*), was assayed by using prephenate as substrate. All enzyme activities shown are expressed as absorbance at 320 nm (see "Materials and Methods"). Lead fractions containing pretyrosine dehydratase and uncontaminated with the prephenate dehydratase·chorismate mutase complex were pooled, concentrated, and chromatographed on hydroxylapatite as described under "Materials and Methods." The resulting separation of three species of pretyrosine dehydratase is shown on the upper right quadrant.

Chorismate Mutase—Two separable activities for chorismate mutase were eluted from DEAE-cellulose (Fig. 7, *lower left*). One chorismate mutase activity co-elutes with prephenate dehydratase as a bifunctional protein (1). The molecular species that passed through the column without retardation is designated chorismate mutase II. The possibility that chorismate mutase II might be a dissociation product of the bifunctional protein was tested by a second passage of the bifunctional protein through the DEAE-cellulose column. The bifunctional protein eluted at its original position, with complete recovery of the total activity applied to the column. These results suggest that an equilibrium dissociation of the mutase/dehydratase protein to yield smaller fragments in identity with chorismate mutase II or pretyrosine dehydratase does not occur. Molecular weights of 134,000 and 27,000 were assigned to the chorismate mutase/prephenate dehydratase protein and to chorismate mutase II, respectively (Table II).

Pretyrosine Dehydratase—The enzymatic conversion of pretyrosine to phenylalanine (pretyrosine dehydratase) constitutes part of one of the two biosynthetic routes to phenylalanine. This enzyme also reacts with prephenate, and for that reason was previously called prephenate dehydratase II (1). The chorismate mutase/prephenate dehydratase protein, on the other hand, does not catalyze the pretyrosine dehydratase reaction. When pretyrosine dehydratase fractions from DEAE-cellulose were pooled, concentrated by Amicon filtration, and chromatographed on hydroxylapatite (Fig. 7, *right*), three stable subforms (*A*, *B*, and *C*) were separated. The

TABLE IV

Substrate specificity of pretyrosine dehydratase subforms

K_m values are expressed as millimolar concentrations, and V_{max} values are expressed as nanomoles per min per mg of partially purified enzyme. Pretyrosine dehydratase activity was assayed at various concentrations of pretyrosine by assay method (b) under "Materials and Methods."

Molecular species[a]	When substrate is:			
	Prephenate		Pretyrosine	
	K_m	V_{max}	K_m	V_{max}
A	0.03	270	0.06	28
B	0.05	333	0.25	50
C	0.05	556	0.25	50

[a] Species A, B, and C of pretyrosine dehydratase were resolved and recovered from hydroxylapatite chromatography as shown in Fig. 6.

TABLE V

Feedback inhibition of biosynthetic enzymes by aromatic metabolites

Preliminary assays were done at several substrate concentrations, especially in the low range of the substrate saturation curve, in order to detect undramatic competitive effects.

Enzyme	Compounds tested as inhibitors (K_i[a])				
	Prephenate	Phenylalanine	Tyrosine	Tryptophan	Pretyrosine
			μM		
Prephenate dehydrogenase		n.i.[b]	35	n.i.	
Pretyrosine dehydrogenase		n.i.	40	n.i.	
Chorismate mutase II	n.i.	n.i.	n.i.	n.i.	n.i.
Chorismate mutase (complex)	40	4	n.i.	n.i.	n.i.
Prephenate dehydratase (complex)		50	—[c]	n.i.	
Pretyrosine dehydratase		n.i.	n.i.	n.i.	
3-Deoxy-D-*arabino*-heptulosonate-7-P synthetase					n.i.

[a] K_i determinations—the apparent K_m (K_{ap}) was determined by plotting the reciprocal of velocity (v^{-1}) against the reciprocal of the substrate concentration (mM^{-1}) at variable concentrations of inhibitor (I). A plot of I as a function of K_{ap} will give $-K_i$ at the intercept of the ordinate. In the cases where no effect was observed, concentrations as high as 0.5 mM potential inhibitor were tested.

[b] n.i., no inhibition.

[c] Activated by tyrosine (see Fig. 8).

original preparation recovered from DEAE-cellulose, as well as each of three subform species, eluted from Sephadex G-200 at a common molecular weight position of 80,000 (Table II). Although some variation in relative ability to react with prephenate or pretyrosine may distinguish the three subform species, these differences are not dramatic (Table IV).

Allosteric Studies—Prephenate/pretyrosine dehydrogenase is inhibited by tyrosine, competitively with respect to the utilization of either substrate (Table V). Consistent with previous findings (1), the chorismate mutase activity of the bifunctional protein was inhibited (competitively with respect to chorismate) by phenylalanine (K_i, 4 μM) and by prephenate (K_i, 40 μM) as shown in Table V. In contrast, the activity of chorismate mutase II was not inhibited by L-phenylalanine, L-tyrosine, or prephenate, even at concentrations as high as 0.5 mM. The prephenate dehydratase activity of the bifunctional

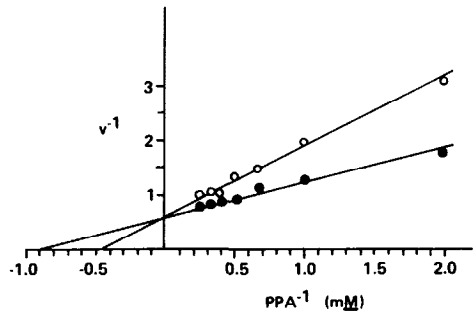


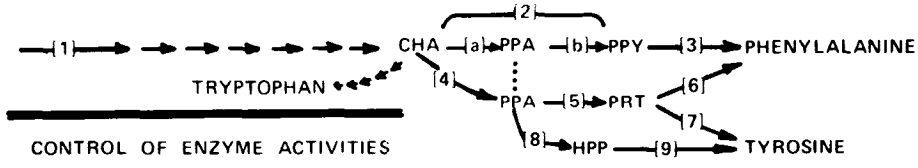
FIG. 8. Activation of prephenate (*PPA*) dehydratase by L-tyrosine. The prephenate dehydratase activity of 50 μl of partially purified chorismate mutase/prephenate dehydratase (recovered from hydroxylapatite chromatography) was determined at the indicated concentrations of prephenate as described under "Materials and Methods," in the presence (●——●) or absence (○——○) of 0.1 mM L-tyrosine. Velocity (v) is expressed as nanomoles of phenylpyruvate formed per min.

protein was inhibited competitively by phenylalanine (K_i, 50 μM). Pretyrosine dehydratase preparations were not inhibited by phenylalanine (Table V) when prephenate was used as substrate. However, the possible inhibition of the enzyme by phenylalanine when pretyrosine is utilized as substrate has not yet been determined, for technical reasons.

Pretyrosine did not inhibit 3-deoxy-D-*arabino*-heptulosonate-7-P synthetase or either of the chorismate mutase activities. The inhibition of 3-deoxy-D-*arabino*-heptulosonate-7-P synthetase by the combination of tyrosine, tryptophan, and phenylpyruvate was described earlier (18). The prephenate dehydratase activity of the chorismate mutase/prephenate dehydratase protein is subject to activation by L-tyrosine as shown in Fig. 8. Tyrosine increases the affinity of the enzyme for substrate; the apparent lack of activation in another experiment can be attributed to the relatively high substrate concentrations that were used (1).

DISCUSSION

Dual Enzyme Routes to Phenylalanine and to Tyrosine—The simultaneous presence of more than one biochemical pathway leading to major end products, such as amino acids, seems unnecessarily wasteful, and the reliance of microorganisms upon single pathways has been a generally acknowledged feature of microbial metabolism. This appeals enormously to generalized concepts of economy and is strongly reinforced by the multitude of experimental observations made in model systems such as *Escherichia coli* or *Bacillus subtilis*. Yet it is becoming increasingly clear that the simultaneous presence of separate pathways leading to a given major metabolite is not so uncommon in nature, and even *E. coli* is not altogether free of such pathway multiplicity (5). The possible significance of metabolic ambiguity for synthesis of small molecules has been discussed elsewhere (4, 5). It seems likely that difficulties encountered in the isolation of amino acid auxotrophs from various groups of bacteria may be explained by the compensatory presence in "silent" mutants of a second pathway to a given end product, intact and unblocked. In such systems mutations may at best produce only leaky phenotypes or perhaps hypersensitivity to inhibition by end product analogues. The enzymological data obtained from wild type *Pseudomonas aeruginosa* and presented in this paper allow an appreciation of our inability to recover, in spite of intensive effort, any tyrosine auxotrophs or nonleaky phenylalanine auxotrophs. The enzyme reactions of aromatic biosyn-



No.	Regulatory Enzyme	Effector(s)	Regulatory Effect	Ref. No.
[1]	DAHP synthetase	PPY, Tyrosine, Tryptophan	Inhibition	17
[2a]	Chorismate mutase	Phenylalanine	Inhibition	1
[2b]	Prephenate dehydratase	Phenylalanine	Inhibition	1
		Tyrosine	Activation	This paper
[6]	Pretyrosine dehydratase	?	?	This paper
[7]	Pretyrosine dehydrogenase	Tyrosine	Inhibition	This paper
[8]	Prephenate dehydrogenase	Tyrosine	Inhibition	9

FIG. 9. Regulation of the dual enzymatic routes to phenylalanine and tyrosine in *Pseudomonas aeruginosa*. *CHA*, chorismate; *PPA*, prephenate; *PPY*, phenylpyruvate; *PRT*, pretyrosine; *HPP*, 4-hydroxyphenylpyruvate; *DAHP*, 3-deoxy-D-*arabino*-heptulosonate-7-P. Unregulated enzymes are designated by number as follows: [*3*], phenylpyruvate transaminase; [*4*], chorismate mutase II; [*5*], prephenate transaminase; [*9*], 4-hydroxyphenylpyruvate transaminase. Prephenate formed on the bifunctional protein may dissociate (shown by *dotted line*) to the free state in the presence of L-phenylalanine (1). Reactions [*7*] and [*8*] may be catalyzed by the same protein. Enzyme [*6*] also possesses prephenate dehydratase activity and was originally called prephenate dehydratase II (1). All four aminotransferases can catalyze Reactions 3, 5, and 9; the relationship of a given aminotransferase to a particular reaction *in vivo* cannot yet be defined.

thesis that have been assayed in *P. aeruginosa* are illustrated in Fig. 9. *E. coli* does not possess the pretyrosine pathways to phenylalanine and tyrosine synthesis (*i.e.* Reactions 5 to 7). The quantitative contributions of the pretyrosine sequence relative to the phenylpyruvate and 4-hydroxyphenylpyruvate routes are uncertain *in vivo* in the absence of suitable mutants and data from radiolabeling experiments. It is assumed that each duality to phenylalanine or to tyrosine which can be demonstrated *in vitro* can be expressed (at least under stress) *in vivo* because mutagenesis procedures that produced many hundreds of other mutations (including tryptophan auxotrophs and shikimate-responsive auxotrophs) have never yielded auxotrophs with absolute requirements for tyrosine or phenylalanine.

Biosynthesis of Pretyrosine—Pretyrosine is formed by transamination of prephenate, a reaction that has not been found in *E. coli* or *B. subtilis* (4). In *Neurospora crassa* prephenate is transaminated *in vivo* only under conditions of anomalous prephenate accumulation (5, 15), and the pretyrosine formed accumulates as a dead-end metabolite. In *P. aeruginosa* four enzymes capable of utilizing prephenate in transamination reactions were isolated. Aminotransferases DE I and HA I possess similar or identical molecular weights, as do aminotransferases HA II and HA III. The overlapping substrate specificities and the molecular weight results suggest the origin of the four aminotransferases from one or two common ancestral proteins via gene duplication and divergence. The collective characteristics of the aromatic aminotransferases show aminotransferases HA II-HA III to share many properties, while the DE I-HA I pair exhibits somewhat more divergent characteristics. All four aminotransferases are exceedingly stable.

Pretyrosine Pathway of L-Phenylalanine Synthesis—Pretyrosine dehydratase is a previously undescribed enzyme which catalyzes the conversion of pretyrosine to phenylalanine. Hence, dual reaction sequences via either phenylpyruvate or pretyrosine exist for phenylalanine biosynthesis in *P. aeruginosa*. Pretyrosine dehydratase, previously named prephenate dehydratase II (1), is most conveniently assayed with prephenate as substrate. Since pretyrosine dehydratase is reactive with prephenate, it seems likely that the prephenate dehydratase component of the bifunctional protein and pretyrosine dehydratase may have evolved from a common ancestral protein. When pretyrosine dehydratase is assayed with prephenate as substrate, no inhibition of activity by phenylalanine occurs. Although the possible inhibition of pretyrosine dehydratase activity by phenylalanine cannot be tested at the moment for technical reasons, the eventual availability of [^{14}C]pretyrosine will make this determination feasible.

Role of Chorismate Mutase II—Two separable activities for chorismate mutase exist. One (*Reaction [2a]*, Fig. 9) is part of a bifunctional protein with prephenate dehydratase; chorismate mutase II ([*4*], Fig. 9) possesses a rather low molecular weight. It seems likely that chorismate mutase II is most significant *in vivo* for tyrosine biosynthesis since the other chorismate mutase activity participates in a channeling mechanism that is specialized for phenylalanine biosynthesis (1).

Prephenate/Pretyrosine Dehydrogenase—Dehydrogenase activities ([*7*] and [*8*], Fig. 9) with either prephenate or pretyrosine as substrate were not resolved into separable proteins. Both reactions exhibit obligate requirements for NAD as the electron acceptor. Nevertheless, we suspect the existence of separate proteins because single mutations have not been found that result in tyrosine auxotrophy. If a single protein were involved, one would expect at least some mutations to abolish both activities. The striking differential effects of *p*CMB (Table III) upon prephenate and pretyrosine dehydrogenase activities are consistent at least with different substrate binding sites.

Regulation of Enzyme Synthesis—Although the enzymes for tryptophan biosynthesis have been shown to be governed by repression control (19), most biosynthetic enzymes in *P. aeruginosa* and other pseudomonads are probably expressed constitutively (5, 20). Ordinarily conclusions about regulation of enzyme synthesis are based upon specific activity data obtained from crude extracts. In crude extracts of *P. aeruginosa*, accurate assay for many of the enzyme activities is complicated by the complex array of competing or interacting enzymes that are present, having overlapping substrate specificities or which may otherwise interfere with technical aspects of a given enzyme assay. Since catabolic aminotransferases of *P. aeruginosa* would probably be regulated by induction mechanisms in the presence of appropriate substrate molecules (20), a comparison of extracts made from a variety of

nutritionally different cultures might provide evidence to equate one or more of the aminotransferases with a degradative activity *in vivo*.

Regulation of Enzyme Activity—An overview of allosteric regulation for aromatic biosynthesis in *P. aeruginosa* is illustrated in Fig. 9. A metabolite from each of the three amino acid branchlets inhibits the activity of 3-deoxy-D-*arabino*-heptulosonate-7-P synthetase in cumulative fashion, and L-tyrosine appears to be the most potent ligand (18). Pretyrosine, a compound which *a priori* might be a logical effector for control of 3-deoxy-D-*arabino*-heptulosonate-7-P synthetase, was not inhibitory to the *P. aeruginosa* enzyme. L-Tyrosine inhibits the activity of pretyrosine dehydrogenase as well as that of prephenate dehydrogenase. L-Phenylalanine inhibits the bifunctional activities, chorismate mutase and prephenate dehydratase, of the protein that channels chorismate to phenylpyruvate (1). In the presence of excess tyrosine and limiting phenylalanine, tyrosine should stimulate phenylalanine synthesis via activation of prephenate dehydratase. In the presence of excess phenylalanine and limiting tyrosine, phenylalanine inhibition of prephenate dehydratase may release enzyme-bound prephenate so that it is free for utilization by enzymes in the flow sequence to tyrosine (1).

Since Enzymes 4 and 5 (Fig. 9) are not subject to inhibition by phenylalanine or tyrosine, one would expect a mutant desensitized for allosteric control of 3-deoxy-D-*arabino*-heptulosonate-7-P synthetase to accumulate pretyrosine. If pretyrosine dehydratase proves not to be inhibited by phenylalanine, then phenylalanine might be overproduced as well. The relatively weak allosteric control of the pretyrosine route of phenylalanine and tyrosine biosynthesis could account for the general ineffectiveness of aromatic analogues as antimetabolites. For example, false feedback inhibition of prephenate dehydratase in the presence of a phenylalanine analogue might result in diversion of the normal flow of chorismate from the phenylpyruvate route to the pretyrosine route.

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